ISOLATION AND AMPLIFICATION OF NUCLEIC ACID MATERIALS

Field of the Invention

The invention relates to the field of purification and amplification of nucleic acids from nucleic acid containing starting materials, especially from biological materials such as urine, faeces, sperm, saliva, whole blood, serum or other body fluids, fractions of such fluids such as leucocyte fractions (buffy coats), cell cultures and the like, but also samples from the environment such as soil, water and the like.

Until recently isolation and/or purification of nucleic acids from complex mixtures as described above was a laborious, multi-step procedure. In EP 0389063, incorporated herein by reference, a simple and rapid purification of nucleic acid material from a complex mixture is disclosed. This procedure comprises treating the complex mixture, such as whole blood, with a chaotropic agent in the presence of a nucleic acid binding silica solid phase material under conditions that allow for binding of all nucleic acid material to said solid phase and separating said solid phase from the mixture. The reference shows that both single stranded and double stranded nucleic acids are bound to the solid phase if present in a mixture. The reference also discloses amplification (PCR) of a certain nucleic acid with a known sequence, suspected to be present in a mixture.

Thus, said reference teaches a simple and rapid detection method for known nucleic acids suspected to be present in a sample.

In many cases the nature of the target nucleic acid (double stranded or single stranded) may not be known beforehand, or there may be many different targets necessary to be analyzed. In these cases the rapid but rather crude method described above may not be sophisticated enough and further separations of the crude material may be desired. Fractionation of mixtures of double- (ds) and single-stranded (ss) nucleic acids (NA) into single- and double-stranded forms is frequently needed e.g. in the separation of labelled ss-NA

probes from ds-hybrids, in the separation of in vitro transcripts from ds-DNA templates, and in the separation of genomic DNA from mRNA. Currently, the separation of different kinds of nucleic acids can be accomplished by several techniques. Electrophoresis can be used to fractionate different forms of nucleic acids, because of differences in size and shape (1-3). Centrifugation takes advantage of differences in density (4), and more recently the technology of high-performence liquid chromatography (HPLC) has been applied to separate and purify single- and double-stranded DNA and RNA molecules (5-8).

RNA purified from eukaryotic cells by the currently most widely used procedure (9) appears to contain significant amounts of genomic DNA. An adaptation which reduces genomic DNA contamination of the ss-RNA fraction has recently been described (10).

It is not possible to look at single stranded and/or double stranded material separately using the method of EP 0389063 because the method does not discriminate between the two.

Summary of the Invention

The present invention, ~~therefor~~ therefore provides a method for separating single stranded nucleic acid material from double stranded nucleic acid material comprising contacting a mixture of the both with a liquid comprising a chaotropic agent and a nucleic acid binding solid phase, whereby the liquid has a composition such that double stranded nucleic acid binds to the solid phase and a substantial amount of single stranded nucleic acid does not and separating the solid phase from the liquid. Suitable circumstances to arrive at such a separation can be determined by the person skilled in the art.

Circumstances under which double stranded material binds to the solid material and single stranded material will vary, however important parameters to obtain such differential binding ar the conc ntration of the chaotropic agent, which should roughly be between 1-10 M, preferably between 3-6 M and particularly about 5 M; the concentration of chelating ag nt, which in the case ~~that EDTA~~ of EDTA is applied should b equal to or

greater than 10 mM and preferably not higher than 1 M; the pH of the aqueous solution in which the separation is carried out should be above 2 when a thiocyanate is used as chaotropic agent and it should be below 10 because otherwise there is a risk that the ds material will become ss. The temperature at which the process is carried out seems to be non-critical, however, it is probably best to keep it between 4°C and 60°C. An important aspect of the process is of course that the ds material remains double stranded during the separation. Under the circumstances as disclosed above this will normally be the case if the ds nucleic acid is at least 50 bp long at 40% GC ~~basepairs~~ base pairs. The skilled artisan knows how this length may vary with lower or higher GC content. In Van Ness et al (26) and/or Thompson et al (27) it is shown that the whole process depends on intricate interactions between ~~a.o.~~ among others the factors mentioned above. Using this disclosure and the cited references the skilled artisan will be able to adjust the circumstances to his or her particular process.

Chaotropic agents are a very important feature of the present invention. They are defined as any substance that can alter the secondary, tertiary and/or quaternary structure of nucleic acids. They should have no substantial ~~effect~~ affect on the primary structure of the nucleic acid. If nucleic acids are present associated with other molecules, such as proteins, these associations can also be altered by the same or different chaotropic agents.Many chaotropic agents are suitable for use in the present invention, such as sodium iodide, potassium iodide, sodium (iso)thiocyanate, urea or guanidinium salts, or combinations thereof. A preferred class of chaotropic agents according to the invention are guanidinium salts, of which guanidinium thiocyanate is most preferred.

By serendipity we found that ss-nucleic acid did not bind to silica particl s or diatomeous earth in th pres nce of buffer L11 (s e examples), whereas ds nucleic acid did. Experiments with different circumstances showed that addition of Mg^{2+} or other positive (bivalent) ions to the unbound

fraction was of great importance. The best r sults were obtained with a concentration of bivalent ion (Mg^{2+}) about equal to the concentration of the chelating agent (EDTA).

The solid phase to be used is less critical. Important is that it should bind nucleic acids reversibly.

Many such materials are known, of which a number are silicium based, such as aluminium silicate and the like, preferably silica. Silica is meant to include SiO_2 crystals and other forms of silicon oxide, such as diatom skeletons, glass powder and/or particles and amorphous silicon oxide. The solid phase may be present in any form, it may even be the vessel which contains the nucleic acid mixtures or a part of such a vessel. It may also be a filter or any other suitable structure. Apart from silicium based materials other materials will also be suitable, such as nitrocellulose (filters), latex particles and other polymeric substances. A preferred form of the solid phase is a particulate form, which allows for easy separation of bound and free material, for instance by centrifugation. The particle size of the solid phase is not critical. Suitable average particle sizes range from about 0.05 to 500 µm. Preferably the range is chosen such that at least 80, preferably 90 % of the particles have a size between the values just mentioned. The same holds true for the preferred ranges of which the average particle sizes are between 0.1 and 200 µm, preferably between 1 and 200 µm. The binding capacity of a given weight of the particles increases with decreasing size, however the lower limit of the size is ~~when~~ that at which particles cannot easily be redispersed after separation through for instance centrifugation. This will be the case in starting material rich in nucleic acids containing many nucleic acids of a higher molecular weight. The particles and the nucleic acids may form aggregates in these cases. The person skilled in the art will be able to choose the right particle siz for the particular application envisioned. The formation of aggregates may be avoid d by using fractionated silica or diatomaceous earth in a number of applications.

A further embodiment of the present invention is a method for isolating single stranded nucleic acid material from a mixture of nucleic acid material, comprising the steps of subjecting the mixture to a method as described hereinabove and treating the supernatant containing the single stranded nucleic acid material with a second liquid comprising a chaotropic agent and a second nucleic acid binding solid phase, whereby the second liquid has a compositon such that the resulting mixture of supernatant and second liquid allow for binding of the single stranded nucleic acid material to the second solid phase.

This way the double stranded nucleic acid material is removed from the crude mixture and the single stranded nucleic acid is purified from the remaining still crude mixture in another single step. Both the double stranded material and the single stranded material are reversibly bound to the respective solid phases, so that they may be easily eluted from said solid phases to undergo further analysis or other treatments. A very useful further treatment is the amplification of the (double or single stranded) nucleic acid material.

Both types can be amplified, or both types may be converted into one another so that they can be amplified. The present invention provides in yet another embodiment a method for amplifying single stranded nucleic acid material comprising the steps of hybridizing the single stranded nucleic acid with primers and elongating the probes using an enzyme which adds nucleotides to the primer sequence using the hybridized single strand material as a template, whereby at least one primer comprises a random hybridizing sequence and an amplification motif.

Single-stranded nucleic acids purified in accordance with the invention were used as input for a cDNA synthesis reaction using primers with random 3' ends (tagging primers) for the first and second strand synthesis (see the outline in Fig. 7).

These tagged cDNAs are then amplified by using only one PCR primer homologous to the PCR motif present in both tagging

primers. The tagging primer used in the first strand synthesis (TAG 20) has been especially designed to facilitate subsequent direct sequencing of the resultant PCR products.

In contrast with most other protocols (16-22) the described method does not need any sequence data at all, and the majority of amplified products can be visualized on ethidium bromide ~~ethidiumbromide~~ stained agarose gels as discrete bands, which makes isolation and direct sequencing of the amplified cDNA feasible. The criteria for amplification are well known in the art. The length of suitable primers, suitable buffers, suitable melting temperatures for separating strands, and suitable hybridization conditions can all be determined using standard handbooks in the field.

Of course the sequences which are exemplified can be varied without departing from the present invention. It is not so much important what sequence is used as an amplification motif, as long as it is suitable for hybridization and primer extension purposes. Suitable limits depend on the conditions which can be varied by the person skilled in the art. Usually primers will be at least 10 bases long and not much longer than 100 bases.

The amplification embodiments of the invention are exemplified using PCR (polymerase chain reaction). Other amplification methods are of course equally suitable.

The exemplified label (or tag) on the primers is DIG (digoxygenin). However other labels are available and well known in the art.

The invention will now be explained in further detail in the following detailed description.

Separation / Isolation

MATERIALS AND METHODS

Source of nucleic acids.

Phage MS-2 ss-RNA (3569 nt), E. coli rRNA (16 and 23S; 1,7kb and 3,5kb respectively), phage M13 ss-DNA (7599 nt) and

HindIII dig sted phage lambda ds-DNA were purchased from Boehringer (Mannheim, Germany). Rotavirus ds-RNA was purified from feces of an infected individual by protocol Y/SC (11). Plasmid DNA was purified from E. coli HB101 as described by Ish-Horowicz and Burke (13) followed by column chromotography with Sepharose CL2B (Pharmacia, Inc. Uppsala, Sweden). Total NA was purified from E.coli by protocol Y/D (11).

Chemicals.

Guanidinium thiocyanate (GuSCN) was obtained from Fluka (Buchs, Switzerland).

EDTA (Titriplex) and MgCl2.6H20 were obtained from Merck (Darmstadt, Germany). TRIS was obtained from Boehringer (Mannheim, Germany). The preparation of size-fractionated silica particles (silica coarse, SC) and diatom suspension has been described (11). Triton X-100 was from Packard (Packard Instrument Co., Inc., Downers Grove, Ill).

Composition of buffers.

The lysis/binding buffer L6, washing buffer L2, and TE (10mM Tris.HCl, 1 mM EDTA; pH=8.0) have been described (11). 0.2M EDTA (pH 8.0) was made by dissolving 37.2 g EDTA (Merck, Germany) and 4.4 g NaOH (Merck, Germany) in aqua in a total volume of 500 ml. Lysis/binding buffer L11 was made by dissolving 120 g of GuSCN in 100 ml 0.2M EDTA (pH=8.0). Binding buffer L10 was prepared by dissolving 120 g GuSCN in 100 ml 0.35M TRIS.HCl (pH 6.4); subsequently 22 ml 0.2M EDTA (pH 8.0) and 9.1 g Triton X-100 were added and the solution was homogenized; finally 11 g of solid $MgCl_2.6H_2O$ was added. The final concentration of $MgCl_2$ in L10 is about 0.25M. L10 is stable for at least 1 month when stored at ambient temperature in the dark.

Fractionation of ds-NA and ss-NA by protocol R.

Th proc dure is outlined in Figure 1. A 50µl specimen (containing a mixture of NA-types in TE buffer) was added to a mixture of 900µl L11 and 40µl SC in an Eppendorf tube and was

subsequently homogenized by vortexing. After 10 min. binding at room temperature, the tube was centrifuged (2 min. at approx. 10.000 x g) which resulted in a silica/ds-NA pellet ("initial silica pellet") and a supernatant containing ss-NA.

To recover ss-NA forms (protocol R-sup), 900µl of the supernatant were added to a mixture of 400µl L10 and 40µl SC and ss-NA was bound during a 10 min. incubation at room temperature. The tube was subsequently centrifuged (15 sec. at approx. 10.000 x g), and the supernatant was discarded (by suction). The resulting pellet was subsequently washed twice with 1 ml of L2, twice with 1 ml ethanol 70% (vol/vol) and once with 1 ml acetone. The silica pellet was dried (10 min. at 56°C with open lid in an Eppendorf heating block) and eluted in 50µl TE buffer (10 min. at 56°C; closed lid). After centrifugation (2 min. at approx. 10.000 x g) the supernatant contains the ss-NA fraction.

To recover ds-NA forms (protocol R-pellet) from the initial ~~silica-pellet~~ silica pellet, the remaining supernatant was discarded, and the silica pellet was washed twice with L11 to remove unbound ss-NA. The resulting silica pellet was subsequently washed twice with L2, twice with ethanol 70%, once with acetone, dried and eluted as described above. After centrifugation (2 min. at approx. 10.000 x g) the supernatant contains the ds-NA fraction.

In the complete procedure (which takes about one hour) for fractionation of NA by protocol R, only two Eppendorf tubes are used.

Fractionation of genomic DNA and ss-NA.

Due to trapping of ss-NA into high-molecular-weight genomic DNA, protocol R as described above gives only low yields of ss-NA. This can be circumvented by first isolating total NA by protocol Y/D (11), which causes some shearing of the high-molecular-weight genomic DNA, sufficient enough to prevent trapping of the ss-NA. Total NA thus purified can subsequ ntly be used as input for protocol R.

G l el ctroph resis.

In all experiments, NA was electrophoresed (8 to 10 V/cm) through neutral agarose slab gels containing ~~ethidiumbromide~~ ethidium bromide (1µg/ml) in the buffer system (40mM TRIS-20 mM sodium acetate-2mM EDTA adjusted to pH 7.7 with acetic acid; ethidium bromide was added to a concentration of 1µg/ml of buffer) described by Aaij and Borst (14).

Hybridization.

DNA fragments were transferred to nitrocellulose filters by the procedure of Southern (15) and hybridized with [alpha-^{32}P]dCTP labelled pHC624 (16) prepared by random labeling (Boehringer, Germany). Hybridization conditions were as described previously (12).

RESULTS

Comparison of different GuSCN-containing ~~lysisbuffers~~ lysis buffers with respect to the binding of different NA-types to silica particles revealed that only doublestranded forms were bound when using L11 (which is about 100 mM for EDTA) as binding buffer; on the other hand both double- and single-stranded forms were bound in binding buffer L6 (which is about 20 mM for EDTA) (Table 1). These observations formed the basis for the development of a protocol (Protocol R) for the fractionation of single-stranded nucleic acids and double-stranded nucleic acids (Fig. 1)

Once double-stranded nucleic acid is bound by silica particles in L11, a brief centrifugation will separate the silica/ds-NA pellet from the supernatant containing the single-stranded forms. Addition of this supernatant to a mixture of silica particles and binding buffer L10 (which is about 250 mM for Mg^{2+}) the binding of single-stranded nucleic acids to the silica particles is restored. Double-stranded and single-stranded forms can subsequ ntly be purifi d by washing and eluting the silica-NA complexes (protocol R). Double-stranded nucleic acid is recovered from the initial ~~silica~~ silica

pellet (protocol R-pellet), whereas single-stranded forms are recovered from the initial supernatant (protocol R-sup).

For optimization of protocol R we performed reconstruction experiments in which previously purified or commercially available, nucleic acids were mixed and subsequently fractionated by protocol R.

Fractionation of a mixture of double-stranded DNA and single-stranded DNA.

The fractionation of a ds-DNA/ss-DNA mixture, into double stranded- and single stranded forms is shown in Figure 2. The recovery estimated from the band intensity of the ethidium bromide stained gel for ss-DNA was about 50%, the estimated recovery of ds-DNA in the range of 500 bp to 4,6 kb was 80%-90% [similar recoveries were obtained for ds-DNA fragments in the range of 100-500 bp (not shown)], larger fragments were significantly sheared as noted before (11). At the level of detection by UV-illumination, fractionation into ds- and ss-forms was complete.

Fractionation of a mixture of double-stranded RNA and single-stranded RNA.

Figure 3 shows the fractionation of a mixture of ds-RNA (human Rotavirus genome segments 1-11; for review see 14) and ss-RNA (phage MS2 RNA) into double stranded- and single stranded forms. The estimated recovery of ds-RNA and ss-RNA was at least 80%. At the level of detection by UV-illumination, fractionation into ds- and ss-forms was complete.

Fractionation of a mixture of double-stranded DNA and single-stranded RNA.

In Figure 4 it is shown that ds-DNA can also efficiently be separated from ss-RNA.

Again are the recoveries for both fractions, at least 80%. Similar results were obtained when E.coli rRNA (23S and 16S) was used as ss-RNA input (not shown).

In the experiments described above, fractionation of ds- and ss-NA forms (as judged by visual inspection of band intensities after ethidium bromide staining and UV illumination) appeared to be complete. In order to establish the performance of the fractionation procedure for a mixture of ds-DNA and ss-RNA into ss- and ds-forms, NA purified by protocol R-sup from such a mixture was studied by Southern blotting and hybridization with a ^{32}P-labelled DNA probe, homologous to the ds-DNA used as input for fractionation. This experiment revealed that the ss-NA fraction contained less than 0.1% of the ds-DNA input (figure 5).

Fractionation of a mixture of genomic DNA and single-stranded RNA.

When we investigated the separation of high-molecular-weight (genomic) dsDNA and ss-RNA by direct fractionation using E. coli as input for protocol R, it appeared that the ds-DNA fraction was heavily contaminated with rRNA (Fig. 6, lanes 6 and 7), and ss-RNA recovery was low (Fig. 6, lanes 8 and 9). This was likely due to trapping of RNA into high-molecular-weight (genomic) ds-DNA when silica/NA complexes were formed. On the other hand no genomic DNA was observed in the ss-RNA fraction. Total nucleic acid, which was first isolated using the standard protocol Y/D (11), and hereafter used as input material in protocol R showed significantly higher recoveries of the ss-RNA fraction (Fig. 6, lanes 2 and 5).

Amplifications

MATERIALS AND METHODS

Source of nucleic acids.

HIV-1 RNA was isolated from a virus culture (23), phage MS-2 RNA was purchased from Boehringer (Mannheim, Germany) and the 7.5 Kb Poly(A)Tailed RNA and the 100 bp ladder used as a marker were purchased from Life Technologies (Gaithersburg, Maryland, USA). The PCR TA3 cloning vector was obtained from Promega (Madison, USA). Plasmids 5' NOT Hxb_2ENN (24) {containing the GAG and POL genes of HIV-1 from nucleotide 638-4647} and 168.1 RTN (24) {containing the ENV gene of HIV-1 from nucleotide 5674-8474} were purified as described by Ish-Horowicz and Burke (13) followed by protocol R-pellet as described in the examples. The plasmid pHCrec used as a positive control in the PCR experiments was made by a low annealing PCR on lambda DNA (Boehringer) using PCR primer RB 8 (see below). The discrete PCR products were purified using protocol Y/D (11) and subsequently cloned in a PCR III vector (Invitrogen) . The revealing plasmid, pHCrec with a approximately 600 bp insert was subsequently purified from E.coli HB101 as described by Ish-Horowicz and Burke (13) followed by column chromotography with Sepharose CL2B (Pharmacia, Inc. Uppsala, Sweden).

Chemicals and enzymes

EDTA, KCl, $MgCl_2.6H_2O$, NaCl and tri-Sodium citrate dihydrate were obtained from Merck (Darmstadt, Germany).TRIS and BSA were obtained from Boehringer (Mannheim, Germany). Triton X-100 was obtained from Packard (Packard Instruments Co., Inc., Downers, Ill, USA). Sodium Dodecylsulfate (SDS) was obtained from Serva (Heidelberg, Germany).

The dNTP's and Dextran Sulphate were obtained from Pharmacia (Uppsala, Sweden).

The chemicals used in protocol R have been described herein.

Reverse transcriptase SuperScript II was purchased from Life Technologies (Gaithersburg, Maryland, USA). DNA polymerase Sequenase 2 was obtained from Amersham (United Kingdom). Ampli-Taq DNA polymerase was obtained from Perkin Elmer (Norwalk, USA). RNAse H was obtained from Boehringer (Mannheim, Germany). Salmon sperm DNA was obtained from Sigma (St. Louis, USA).

Composition of buffers and solutions.

The preparation of the buffers used in protocol R have been described herein, except that the lysis buffer and washing buffers (L10, L11, and L2) used in protocol R for the isolation of nucleic acids were filtered through a column packed with Diatoms (11) in order to remove any endogenous nucleic acids in the lysis buffer and washing buffers.

Sub B4 The 10 x reverse transcription buffer (CMB1) consists of 100 mM Tris.HCl (pH 8.5), 500 mM KCl and 1% Triton X-100.

The 10 x PCR buffer consists of 500 mM Tris.HCl (pH 8.3), 200 mM KCl and 1 mg/ml BSA.

The elution buffer Tris/EDTA (TE, pH 8.0) consists of 10 mM Tris.HCl (pH 8.0) and 1 mM EDTA (pH 8.0).

Oligonucleotides.

Sub B5 The first strand primer **TAG 20:**

5'GACAGAATGCCGAAATGACCCCNNNNNG3'

The second strand primer **TAG 7:**

5'DIG-GACAGAATGCCGAAATGANNNNNG3'

The PCR primer RB 8:

5'GACAGAATGCCGAAATGA3'

underlined: PCR motif
bold: motif for direct sequencing
N= A, T, C, or G

Protocol for first strand synthesis.

ss-RNA, present in the commercially available reverse transcriptases, appeared to produce unwanted side products when used in first strand synthesis. To overcome this problem reverse transcriptase was first pretreated in a mixture for cDNA synthesis lacking exogenously added primers:

1 µl SuperScript II (200 U/µl)
1 µl CMB1 (10 x)
0.5 µL $MgCl_2$ (100 mM)
0.4 µL dNTP's (25 µM each)
7.1 µL H_2O
Incubate 15 min. at 37°C

Nucleic acids (20 µl) purified by protocol R-sup were incubated for 5 min. at 60°C and thereafter quenched on ice. Subsequently the following mixture was added:

3 µl CMB1 (10 x)
1 µl TAG 20 (100 ng/µl)
1.5 µL $MgCl_2$ (100 mM)
1.2 µl dNTP's (25 mM each)
3.3 µl H_2O

Finally 10 µl of the preincubated Superscript II (SS II) was added and the resulting mixture was incubated for 30 min. at 42°C.

After the reverse transcription reaction SS II was inactivated by incubating the mixture for 5 min. at 80°C, and the mixture was subsequently cooled down to room temperature. In ord r to convert the RNA/DNA hybrids into single-stranded cDNA twenty units of RNAse H were added to the mixture and incubated for 60 min. at 37°C. The single-stranded cDNA was subsequently isolat d using protocol R-sup. The single-

stranded cDNA was eluted in 40 µl TE and 20 µl was used as input for second strand synthesis.

Protocol for second strand synthesis.

To twenty ~~microliter~~ microliters of single-stranded cDNA, the following mixture was added (on ice):

4 µl	CMB1	(10 x)
1 µl	TAG 7-DIG*	(100 ng/µl)
2 µl	$MgCl_2$	(100 mM)
1.6 µl	dNTP's	(25 mM each)
0.2 µl	Sequenase 2	(13 U/µl)
11.2 µl	H_2O	

The mixture was incubated for 10 min. on ice, and subsequently for 60 min. at 37°C. After the second strand synthesis the double-stranded cDNA was isolated using protocol R-pellet. The double-stranded cDNA was eluted in 40 µl TE. ~~Twenty microliter was taken of~~ Twenty microliters was taken off and 2 µl was used as input for PCR. The remaining 18 µl was stored at -20°C.

Protocol for the polymerase chain reaction.

Two microliters of double-stranded cDNA was added to 48 µl of a PCR mixture consisting of:

18 µl	TE	(pH 8.0)
1 µl	RB 8	(100 ng/µl)
5 µl	PCR buffer	(10 x)
0.9 µl	$MgCl_2$	(100 mM)
0.2 µl	dNTP's	(100 µM)
0.1 µl	dUTP*	(25 µM)
0.3 µl	Ampli Taq	(5 U/µl)
22.5 µl	H_2O	

After incubation for 5 min. at 95°C the sample was subjected to 45 cycles of amplification in a DNA thermal cycler (type 480; Perkin Elm r Cetus). A cycle consisted of

denaturation for 1 min. at 95°C, annealing for 1 min. at 63°C, and elongation for 2 min. at 72°C. After cycling the sample was incubated for 8 min. at 72°C, and subsequently the temperature was lowered to 4°C. ~~Twentyfive~~ Twenty-five microliters of the PCR product was examined by agarose gel electrophoresis and ~~ethidiumbromide~~ ethidium bromide staining. In every experiment TE was used as a negative extraction control and as a negative PCR control.

*Partial substitution of dTTP with dUTP provides a methodology for ensuring that products from previous PCR reactions cannot be reamplified. Products of PCR amplifications will be uracil-containing deoxyribonucleic acids. Possible contaminating PCR products from a previous PCR amplification will be eliminated by excising uracil bases using the enzyme Uracil N-glycosylase (UNG) prior to PCR (25)

Gel electrophoresis.

In all experiments, the nucleic acids were electrophoresed (8 to 10 V/cm) through neutral agarose slab gels containing ~~ethidiumbromide~~ ethidium bromide (1 µg/ml) in the buffer system as described by Aaij and Borst (14)

Hybridization.

DNA fragments were detected after Southern blotting (15) by hybridization with ^{32}P-labelled probes representing the entire GAG, POL, and ENV genes of HIV-1 [plasmid 5' NOT Hxb_2ENN and plasmid 168 1 RTN](10).

RESULTS

In parallel, 10^5 molecules of HIV-1 RNA (23) and negative controls (TE) were extracted using protocol R-sup. The resulting single-stranded nucleic acids were amplified by the non-selective RT-PCR as disclosed above, resulting in a discrete banding pattern for HIV-1 RNA, and no amplification products in the TE controls (Fig. 8). Th variation between the duplicates is a reflection of the non-selectivity of the procedure. As a control for the efficiency of the PCR part of

the procedur we used an input of 0, 6, 60 and 600 molecules of the plasmid pHCrec.

In order to confirm the ~~HIV-origin~~ HIV origin of the bands visible in figure 8 A, we performed a Southern blot hybridization under high stringency conditions with ^{32}P-labelled probes encompassing almost the entire HIV-1 genome (Fig. 8 B). This experiment showed that most of the bands visible by UV-illumination hybridized with the HIV-1 probe. The bands that did not hybridize with the probe might be homologous to parts of the HIV-1 genome other than those present in the probe or might originate from single-stranded RNA present in the HIV-1 RNA preparation (e.g. cellular mRNA) or ss-RNA present in Superscript II, which was not converted to ds-hybrids during the preincubation of the SuperScript II.

Similar results were obtained with other single-stranded RNAs such as hepatitis C virus RNA, phage MS2 RNA, and the 7.5 Kb Poly(A)-Tailed RNA (results not shown).

It is concluded that the described procedure can be used to amplify single stranded RNA targets (present in e.g. serum) to a series of discrete bands in agarose gels. The discrete bands can be purified from agarose gels, cloned in e.g. a bacterial vector and the clones can subsequently be sequenced. Due to the fact that one of the tagging primers (TAG 20) harbours a sequence motif it is possible to sequence the discrete bands without cloning, after the bands are purified from gel. The method described here is useful in isolating and characterizing unknown sequences present in clinical samples (e.g. viral sequences) or for the amplification of cDNAs from transcripts without having any sequence data.

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NA-type	binding in L6	binding in L11
ds-DNA	+	+
ds-RNA	+	+
ss-DNA	+	–
ss-RNA	+	–

Table 1.

Binding of different NA-types to silica particles in different lysisbuffers; similar results were obtained using diatoms rather than silica particles (data not shown).